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(71) Applicants
Sankyo Company,
Limited,
No. 1-6, 3-chome,
Nihonbashi Honcho
Chuo-ku, Tokyo, Japan
(72) Inventors
Rei Matsueda
Yoshio Iizuka
Kenichi Shinkai
(74) Agents
Marks & Clerk,
57-60 Lincoln's Inn Fields,
London, WC2A 3LS

(54) **Hydrolyzed polysaccharide**

(57) A glucan having β-(1 → 3) bonding in its principal chain and β-(1 → 6) bonding in its branches is prepared by treating a polysaccharid produced by a fungus of the family *Corticiaceae* with formic acid and then hydrolyzing the resulting product. This hydrolyzed glucan has valuable immunomodulatory properties and thus is useful in the treatment of cancers and articular rheumatism.

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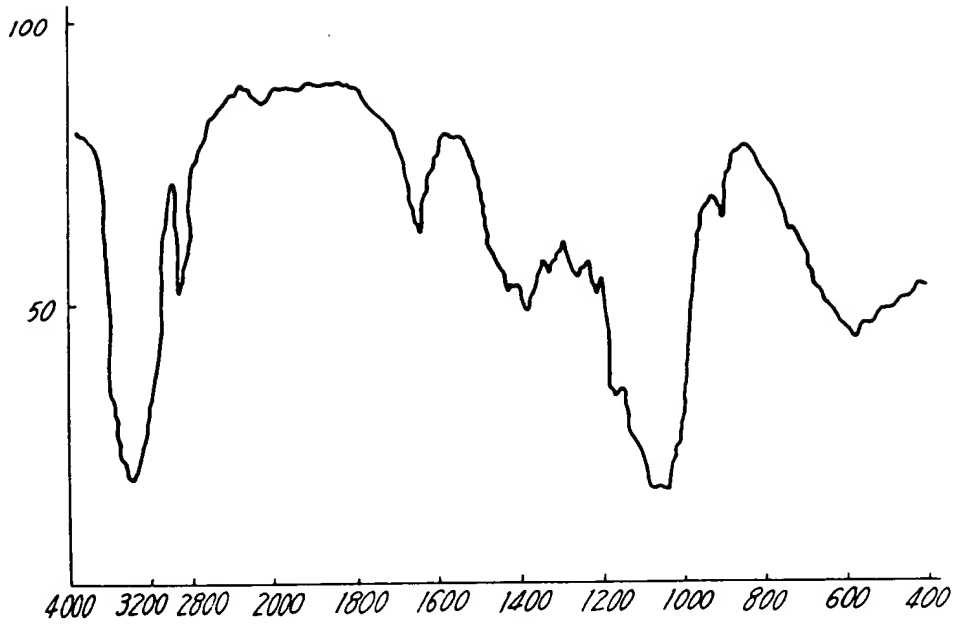
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SPECIFICATION

**Hydrolyzed polysaccharide**

The present invention relates to a new hydrolyzed glucan, to a process for its preparation and to pharmaceutically acceptable compositions containing it. The glucan of the invention is useful as an immunomodulatory agent in the treatment of cancers and of articular rheumatism.

It is well-known that fungi of the class Basidiomycetes can produce polysaccharides which have activity against cancers and other tumours. However, these polysaccharides are sparingly soluble in water and, even if it is possible to produce an aqueous solution of any of them, this solution will readily gel. As a result, it is very difficult to carry out pharmacological experiments with these polysaccharides and even more difficult to use the polysaccharides therapeutically.

Articular rheumatism is an incurable disease and, although many drugs have been investigated in an attempt to cure it, they have not been successful. Those treatments which are known (for example, treatment with steroids, non-steroid anti-inflammatory agents, gold drugs etc.) are all symptomatic treatments, not causal treatments.

Japanese Patent No. 828,248 describes a process for producing a polysaccharide, which has anti-tumour activity, by cultivating a glucan-producing microorganism of the genus *Corticium* or the genus *Hypchnus* and then separating from the culture broth a glucan having $\beta$-(1→3) bonds in its main chain and $\beta$-(1→6) bonds in its branches. However, as described above, the resulting glucan, although having anti-tumour activity, is difficult to use in practice because its aqueous solutions tend to gel. We have, however, now surprisingly found that, by treating glucans of this type with formic acid and then hydrolyzing the resulting product, we can produce a glucan having substantially improved solubility in water and, moreover, quite unexpectedly, very much improved anti-tumour activity.

The present invention thus provides a glucan having $\beta$-(1→3) bonds in its main chain and $\beta$-(1→6) bonds in its branches, the ratio of glucose units in the branches to glucose units in the main chain being about 2 : 7, the glucan being characterized by the following properties:

(a) the freeze-dried product is a white, amorphous solid;

(b) it is soluble in water, in dimethyl sulphoxide and in dimethylformamide, and is insoluble in ethanol, acetone, ethyl acetate, benzene and diethyl ether;

(c) its elemental analysis essentially corresponds to the values calculated for a hexoside-type polysaccharide with bound water for formula $(C_6H_{10}O_5)_n \cdot nH_2O$ [in which $n$ is a number, calculated from property (e), exceeding 800];

(d) it has an infrared spectrum (KBr powder) essentially corresponding to that shown in the accompanying drawing; and

(e) a single peak only is observed on analytical ultracentrifugal spectroscopy, and it has a molecular weight from 150,000 to 160,000, calculated from the sedimentation constant;

and the glucan being capable of preparation by treating a polysaccharide produced by a fungus of the family *Corticiaceae* with formic acid and hydrolyzing the treated product.

The invention also provides a process for preparing the hydrolyzed glucan of the invention by treating a polysaccharide produced by a fungus of the family *Corticiaceae* with formic acid and then hydrolyzing the treated product.

The invention still further provides a pharmaceutical composition comprising the hydrolyzed glucan of the present invention in admixture with a pharmaceutically acceptable carrier or diluent.

The polysaccharide employed as the starting material for the process of the invention is a metabolic product of a fungus of the basidiomycetous family *Corticiaceae,* for example a fungus of the genus *Corticium* or *Hypochnus*. This starting polysaccharid may be produced by cultivating the chosen fungus as described in more detail in Japanese Patent No. 828,248.

The hydrolyzed glucan of the present invention is then preferably obtained by adding 70 - 90% w/w aqueous formic acid to the starting polysaccharide and heating the resulting mixture, preferably with stirring. The temperature to which the reaction mixture is heated is not particularly critical, although a temperature of from 80°C to 100°C is preferred. Th time required for this reaction will depend upon the reaction conditions, especially the temperature, but the reaction will normally be complete within a period of from 5 minutes to 1 hour. We prefer that excess formic acid should then be removed by evaporation under reduced pressure. Water is then added to hydrolyze the residue. The hydrolysis is preferably effected by heating the mixture under reflux until deformylation is complete. The gelatinous material produced may then be removed by centrifugation and an organic solvent is added to the mother liquor to produce a precipitate, which is then collected. There is not particular limitation on the nature of the solvent used in this stage, provided that it does not dissolve the hydrolyzed polysaccharide; and alcohol, such as methanol or ethanol, is preferred. This precipitate is then dissolved or dispersed in water and the solution or dispersion is freeze-dried to give the desired hydrolyzed polysaccharide in the form of a white, amorphous solid.

This amorphous solid is soluble in water, in dimethyl sulphoxide and in dimethylformamide and is insoluble in ethanol, acetone, ethyl acetate, benzene and diethyl ether. It is resistant to heat. Its elemental analytical data corresponds to that calculate for a hexoside-type polysaccharide containing bound water and having the formula $(C_6H_{10}O_5)_n \cdot nH_2O$. The infrared spectrum of the product (in potassium bromide powder) is illustrated in Figure 1. It has a specific rotation $[\alpha]_D^{20} = -15° \pm 5°$ (c = 0.1, dimethyl sulphoxide). Only a single peak was observed in an analytical ultra-centrifugal spectrum of the product and the molecular weight was found, from the sedimentation constant, to range from 150,000 to 160,000, from which it is possible to calcu-

late the value of $n$ in the above formula as being from 833 to 889.

The constituent saccharides in this hydrolyzed glucan can be identified as follows. The hydrolyzed glucan is dissolved in 1N aqueous sulphuric acid and then further hydrolyzed at 100°C for 120 minutes in a sealed tube. The product is then neutralized with barium hydroxide and subjected to paper chromatography. On developing the chromatography paper with an aqueous ammonical solution of silver nitrate, a single spot only is observed. The product represented by this single spot can be identified as glucose by comparing it with a repeat experiment using a standard sample. The types of bonding of the glucose units making up the hydrolyzed glucan of the present invention can be determined by completely methylating the glucan by conventional means, hydrolyzing the methylated glucan to give methylated glucose, reducing the methylated glucose and acetylating the reduced product. Gas chromatography identified the resulting mixture as acetates of 2,3,4,6 - tetra - *O* - methylglycitol, 2,4,6 - tri - O - methylglycitol and 2,4 - di - O - methylglycitol in a molar ratio of approximately 1 : 2.5 : 1. This result confirms that the glucan of the present invention contains about two branches (each of a single glucose unit) bonded with (1 → 6) bonding for each 7 glucose units, having (1 → 3) bonding, in the glucan main chain. Furthermore, it was confirmed that all of the glucose bonds were in the $\beta$ configuration, by observation of products decomposed with endo - $\beta$ - 1,3 - glucanase and exo - $\beta$ - 1,3 - glucanase.

The hydrolyzed glucan of the present invention is more soluble in water and less liable to undergo gelling in aqueous solution than is the polysaccharide from which it was prepared. Moreover, its immunomodulatory activities (e.g. anti-cancer activity and arthritis suppressive activity) are very strong and accompanied by a low toxicity. In particular, the strong arthritis suppressive activity and low toxicity could not have been anticipated from the activities of polysaccharides produced directly from fungi of the class Basidiomycetes. Accordingly, the hydrolyzed glucan of the invention is useful for treating diseases resulting from disorders of the immune system, .g. articular rheumatism.

The biological activities and toxicity of the hydrolyzed glucan of the invention are demonstrated by the following tests.

(1) *Anti-neoplastic activity*

These experiments were carried out on male mice, 7 weeks old, of the ICR strain. $2 \times 10^6$ Sarcoma 180 cancerous cells are grafted onto the axillary skin of each mouse. 6 and 7 days after grafting, a sample of the hydrolyzed glucan of the invention (produced as described in the following Example 1) in sterilized physiological saline was administered by injection into the abdominal cavity; the concentration of the saline solution of hydrolyzed glucan was 0.1% w/v. 25 days after the grafting, the diameter of the tumour was measured. The experiment was repeated using a control group of mice to which no hydrolyzed glucan was administered and the tumour suppressive ratio (%) was calculated from the following formula:

$$\frac{d_o - d}{d_o} \times 100$$

in which $d_o$ is the average tumour diameter (mm) of the control group and d is the average diameter of the treated group. The results are given in Table 1, which shows the effect on the tumour suppressive ratio of varying the amount of hydrolyzed glucan administered per day.

45 days after the grafting all of the mice treated were carefully examined to determine the number of mice showing complete regression of the tumour. The results of this examination are also shown in Table 1, in which the number of mice showing complete regression is reported as a proportion of the total number of mice tested in the relevant group.

*Table 1*

| *Amount administered (mg/kg body weight/day)* | *Tumour suppressive ratio (%)* | *Complete regression* |
|---|---|---|
| 0.1 | 47 | 1/5 |
| 1.0 | 85 | 3/4 |
| 10.0 | 100 | 5/5 |

(2) *Suppressive activity against arthritis*

These experiments were carried out following the procedures of Winder *et al* [C.V. Winder, L.A. Lembke and M.D. Stephens, Arth. Rheum., *12*, 472 (1969)], which measure the ability of compounds to suppress adjuvant-induced arthritis of rats, this experiment being conventionally employed for the evaluration of anti-neoplastic agents.

Lewis rats were given a subcutaneous injection of the adjuvant in the plantar surface of each right hind foot to induce the disease. A solution of the hydrolyzed glucan of the invention was then injected daily into the abdominal cavity for 15 days following innoculation of the adjuvant. The experiments wer repeated with a control group of animals to which the hydrolyzed glucan was not administered. On the 18th, 20th, 24th, 28th and 32nd days after innoculation of the adjuvant, the swellings of the feet of the treated rats were compared with the swellings of the feet of the untreated rats and, using a formula similar to that used to calculate the tumour suppressive ratio, a suppressive ratio was calculated for each dose of the hydrolyzed glucan. The results are given as averages in the following Table 2.

*Table 2*

| *Amount administered (mg/kg body weight/day)* | *No. of animals* | *Suppressive ratio* | *$ID_{50}$* |
|---|---|---|---|
| 0.1 | 5 | 36.8 ± 3.6 | 0.33 |
| 1.0 | 10 | 59.0 ± 3.9 | |

(3) *Acute toxicity*

An acute toxicity test (one week observation) was carried out by injecting samples of the hydrolyzed glucan into the abdominal cavity of male *ddy* mice. After administering samples in amounts of 100 and 300 mg/kg, no deaths were observed and body weight increased normally.

As is apparent from the results described above, the hydrolyzed glucan of the present invention is of considerable value as an immunomodlatory agent. Administration of the agent is preferably effected parenterally, e.g. by subcutaneous injection, intravenous injection or intramuscular injection. The daily dosage will vary depending upon the disease to be treated, the route of administration and the frequency of administration, but, in general, the adult daily dosage is preferably from 0.5 to 50 mg, e.g. about 5 mg. This may be administered as a single dose or in divided doses.

The hydrolyzed glucan of the invention can be prepared in a form suitable for the chosen route of administration, using any of the formulations commonly used for other immunomodulatory agents. For example, a composition can be provided in an ampoule in a unit dosage amount or it may be provided in a multiple dosage container, preferably together with an antiseptic substance. The composition can be in the form of a suspension, a solution or an emulsion in an oily or aqueous vehicle and can include conventional adjuvants, for example suspending agents and/or stabilizers and/or dispersing agents. Alternatively, the active ingredient can be provided in the form of a powder which is dissolved prior to administration in an appropriate vehicle, for example sterile, pyrogen-free water.

If the composition of the invention is provided in unit dosage form, it preferably contains from 0.5 to 10 mg of the active ingredient per unit dose.

The invention is further illustrated by the following Examples, of which Example 1 illustrates the preparation of the hydrolyzed glucan of the invention and Example 2 illustrates the preparation of a pharmaceutical composition containing it.

*EXAMPLE 1*

To 3 g of Corticane (a polysaccharide produced by a fungus of the family *Corticiaceae*) were added 240 ml of 90% w/w aqueous formic acid; the mixture was heated to 95°C for 20 minutes, with stirring. After evaporating off the formic acid under reduced pressure, 600 ml of water were added to the residue and the resulting aqueous mixture was heated under reflux. The reaction mixture was then centrifuged for 15 minutes at 10,000 rpm. To the supernatant thus separated were added 2.4 litres of ethanol to produce a precipitate, which was then collected by centrifugation. 500 ml of water were added to the precipitate and then the aqueous mixture was freeze-dried to give 2.0 g of the desired hydrolyzed glucan in the form of a white amorphous solid. The product had the following properties:

Elemental Analysis

Calculated for $(C_6H_{10}O_5)_n.nH_2O$:

C, 40.00%; H, 6.71%; water 10.00%.

Found: C, 40.22%; H, 6.52%; water, 9.63%.

Specific rotation:

$[\alpha]_D^{20} = -15°$ (c = 0.1, dimethyl sulphoxide).

Infrared Absorption Spectrum (KBr) $\nu cm^{-1}$:

3400, 1640, 1080, 1040.

Molecular weight (analytical ultra-centrifugal determination, phosphate buffer, pH 6.5): 159,000.

*EXAMPLE 2*

5 mg of the hydrolyzed glucan obtained in Example 1 were dissolved in 2 ml of physiological saline and then the solution was sterilized by heating in the usual way to provide an injectible solution.

*PREPARATION*

Into each of five 500 ml. Sakaguchi flasks were introduced 100 ml. of a glucose-potato medium (containing 2% w/w of glucose in a boiled soup containing 200 g/litre of potato), and the contents were sterilized. A culture of *Corticium vagum* F-31-9 (FERM No. 302) was then incoluated and shaking cultivation was conducted at 26°C. for 8 days. After completion of the cultivation, 800 ml. of distilled water were added to 400 ml of the culture broth and the pH of the mixture was adjusted to a value of 7.4. The mixture was then homogenized in a homogenizer and the solid substance was removed by centrifugal separation.

To 1 litre of the supernatant were added 3 litres of ethanol, and the resulting precipitate was collected by centrifugation and dissolved in distilled water. Subsequently, 162 ml of 0.1 M aqueous cetyltrimethylammonium bromide and 13 ml of 0.5M aqueous sodium hydroxide were added thereto to adjust the pH value of the solution to 12.6, thus precipitating polysaccharides. 300 ml of 10% w/v aqueous acetic acid were added to the resulting precipitate to dissolve it and then 1.2 litres of ethanol were added to the solution. The resulting precipitate was dissolved in 350 ml. of distilled water and the solution was introduced into a cellulose tube and dialysed against distilled water. To 500 ml of the dialysate were added 2 litres of ethanol to precipitat again the polysaccharides. The resulting precipitate was dissolved in 100 ml. of distilled water and then freeze-dried to afford 2 g of a crude glucan.

13.5 mg of the crude glucan thus obtain were dissolved in 27 ml of distilled water and the solution was passed through a column of Sephadex G-200

(Sephadex is a trade mark). The column was then eluted with distilled water and the eluate collected in 20 ml fractions. The glucan showed a peak in a third fraction and 75.3% of the total polysaccharide was recovered in fractions 2 to 11 inclusive. Fractions 2 to 11 were combined and 800 ml of ethanol were added to the resulting solution to cause precipitation. The resulting precipitate was dissolved in distilled water and then freeze-dried to afford 9.2 mg of a glucan, which was named as "Corticane". Corticane is a white neutral substance.

CLAIMS

1. A hydrolyzed glucan having $\beta$-(1→3) bonds in its main chain and $\beta$-(1→6) bonds in its branches, the ratio of glucose units in the branches to glucose units in the main chain being about 2 : 7, the glucan being characterized by the following properties:
   (a) the freeze-dried product is a white, amorphous solid;
   (b) it is soluble in water, in dimethyl sulphoxide and in dimethylformamide, and is insoluble in ethanol, acetone, ethyl acetate, benzene and diethyl ether;
   (c) its elemental analysis essentially corresponds to the values calculated for a hexoside-type polysaccharide with bound water of formula $(C_6H_{10}O_5)_n.nH_2O$ [in which $n$ is a number, calculated from property (e), exceeding 800].
   (d) it has an infrared spectrum (KBr powder) essentially corresponding to that shown in the accompanying drawing; and
   (e) a single peak only is observed on analytical ultra-centrifugal spectroscopy, and it has a molecular weight from 150,000 to 160,000, calculated from the sedimentation constant;

and the glucan being capable of preparation by treating a polysaccharide produced by a fungus of the family *Corticiaceae* with formic acid and hydrolyzing the treated product.

2. A process for preparing a hydrolyzed glucan by treating a polysaccharide produced by a fungus of the family *Corticiaceae* with formic acid and then hydrolyzing the treated product.

3. A process according to Claim 2, in which the formic acid employed is 70 - 90% w/w aqueous formic acid.

4. A process according to Claim 2 or Claim 3, in which the treatment with formic acid is effected at a temperature of from 80°C to 100°C.

5. A process according to any one of Claims 2, 3 and 4, in which hydrolysis is effected by heating a mixture of water and said treated product under reflux until deformylation is complete.

6. A process according to Claim 2, substantially as hereinbefore described with reference to foregoing Example 1.

7. A hydrolyzed glucan when produced by a process according to any one of Claim 2 to 6.

8. A pharmaceutical composition comprising a hydrolyzed glucan according to Claim 1 or Claim 7 in admixture with a pharmaceutically acceptable carrier or diluent.

9. A pharmaceutical composition according to Claim 8, formulated for parenteral administration.

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